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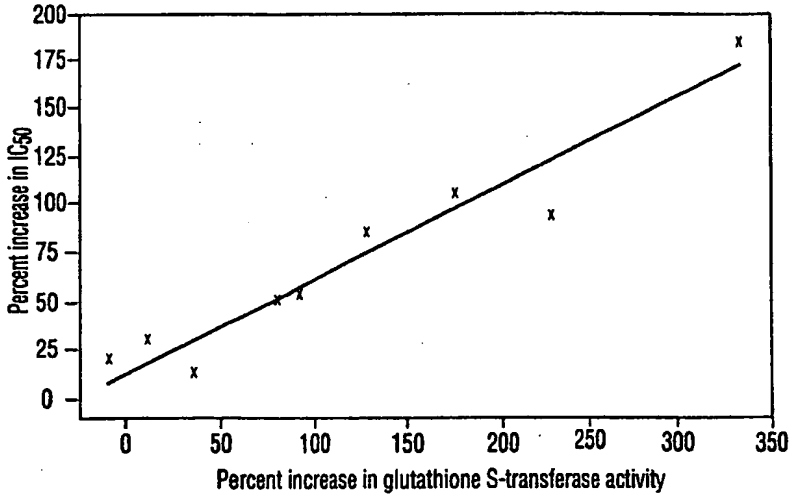

(57) Abstract

Compounds that increase the specific activity of isoforms of glutathione S–transferase are useful in protecting cells against the toxic effects of oxygen–containing free radicals in mammals. Thus, the invention provides a method of treating diseases or cellular damage caused by oxygen–containing free radicals, or of preventing or minimising such damage, which comprises administering to a patient in need thereof an effective amount of one or more glutathione S–transferase elevating compounds. The compounds may also be used for protecting healthy cells during cancer treatments that generate oxygen–containing free radicals in tissues. Such compounds consist of Michael reaction acceptors, compounds convertible to Michael reaction acceptors by oxidation or metabolism, halogenated compounds subject to nucleophilic displacement reaction via glutathione S–transferase, 1,2–dithiol–3–thiones, triphenols with vicinal hydroxyl groups, nifuroxime, and vitamin K–S(II).

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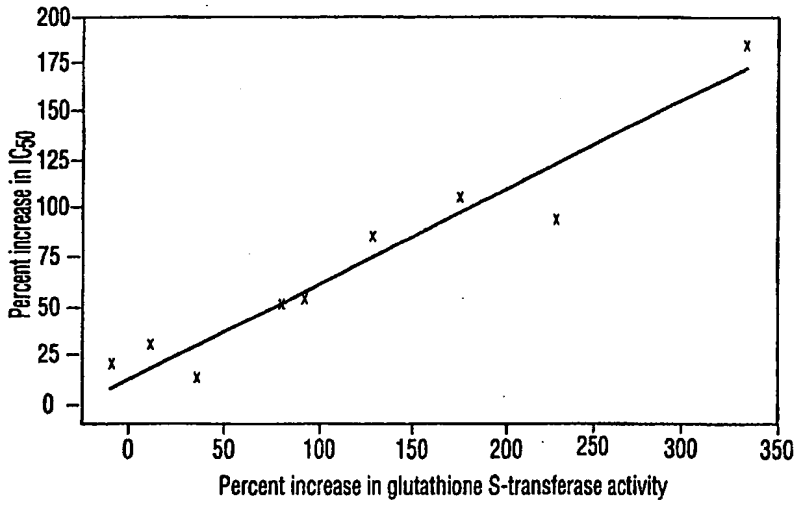

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TREATMENT OF DISEASES OR PREVENTION OF CELLULAR DAMAGE CAUSED BY OXYGEN-CONTAINING FREE RADICALS

TECHNICAL FIELD

This invention relates to a method of treating or preventing disease or cellular damage in mammals in which oxygen-containing free radicals play a pathological role. The method depends on treatment with compounds that increase the activity of an enzyme that protects cells against the damaging effects of free radicals.

BACKGROUND ART

Damage by oxygen-containing free radicals is implicated in the pathology of a number of diseases. These include conditions associated with an inflammatory component (e.g. rheumatoid arthritis, psoriasis), and ischemia-reperfusion diseases (e.g. stroke, brain injury, heart attack, and cerebral damage after cardial arrest). Free radical damage is implicated in several neurological conditions (e.g. Alzheimer's disease, Parkinson's disease, schizophrenia, tardive dyskinesia) and in other conditions (e.g. atherosclerosis, cataracts, ulcerative colitis, cancer, and AIDS). Other medical situations in which damage due to oxygen-containing free radicals is implicated are sunburn, overexposure to therapeutic radiation, and ischemia-reperfusion associated with surgical procedures (e.g. cardiopulmonary bypass, microsurgery, intraaortic balloon pump insertion, aneurism repair, dissection of emboli, organ transplantation). Free radical damage is also implicated in aging.

The oxygen-containing free radicals associated with disease are produced in cells during the course of normal oxidative metabolism. Cells are normally protected against free radical damage by various defense mechanisms. Pathological free radical damage occurs under conditions

where the rate of radical production exceeds the capacity of the defense mechanisms.

The hydroxyl radical is particular damaging to cells because it reacts extremely rapidly with virtually all cell constituents. In addition to damaging cellular constituents, reactions of the radical lead to products which react with and cause additional damage to cell constituents.

The hydroxyl radical can be produced from hydrogen peroxide, a normal constituent of cells, by reaction with certain transition metal ions present in cells. Other possible sources relevant to cells are the reaction of superoxide with nitric oxide, the reaction of hypochlorous acid with superoxide, and exposure to ionising radiation.

Free radical-damaged fatty acids of the type produced by the hydroxyl radical can also be produced by reactions in cells of arachidonic acid, which can accumulate in tissues as the result of ischemia. Action of the hydroxyl radical on moieties bearing certain fatty acids and enzymic reactions of arachidonic acid can both lead to the formation of peroxyl radicals, an intermediate in free radical damage of cellular lipids.

Aspects of cellular defenses against the hydroxyl radical can be grouped as operating either prior to the formation of the radical or after it has damaged cell components. An example of defenses that operate prior to formation of the hydroxyl radical are enzymes that keep the concentration of hydrogen peroxide low by catalyzing its breakdown to innocuous materials. Defenses after formation and reaction of the hydroxyl radical act in a damage-control capacity. These defenses consist of antioxidants that scavenge free radicals and an array of enzymes. The antioxidants halt free radical lipid chain reactions. The enzymes carry out many different functions such as the repair and replacement of damaged cell

constituents, and the detoxification of radical-produced toxins. A wide variety of enzymes are involved, in part, because of the many different molecules damaged by the hydroxyl radical, for example proteins, lipids and DNA. Damage-control enzyme activities related to lipids include the detoxification of lipid hydroperoxides, aldehydes produced by the decomposition of radical-damaged lipids and epoxidized lipids produced by free radical action.

An approach to treat free radical diseases in which damage by the hydroxyl radical is pathological would consist of the administration of compounds that increase the specific activity of damage-control enzymes by enzyme induction or other mechanisms. These compounds could also be used to treat diseases where damage similar to that caused by the hydroxyl radical is caused by other agents, such as arachidonic acid and certain anti-cancer agents. The compounds could be administered either acutely, that is when free radical damage is occurring or has occurred or prophylactically. Because many enzymes are involved in damage-control following hydroxyl radical damage, the effectiveness of the approach will depend on increasing the specific activity of those particular enzymes that play critical roles in coping with the damage. A characteristic of critical enzymes in the present context is that they are present in cells at levels sufficient to cope with low levels of free radicals, but not with the high levels that lead to pathological damage. An experimental criterion of a critical enzyme is an increase in resistance to oxygen-containing free radicals in proportion to increases in activity of the enzyme.

Previously, compounds that increase the specific activity of critical enzymes that act in a damage-control capacity in protecting against the cytotoxic effects of a physiological source of the hydroxyl radical, such as hydrogen peroxide, had not been identified. The prior art

for the use of drugs to increase the level of enzymes that protect against free radical damage does not deal with induction of enzymes with damage control functions. Kerr et al (U.S. Patent No. 5,001,141) describe compounds useful for protecting against oxygen-derived free radical damage in mammals. These compounds increase the level of the enzymes superoxide dismutase, and catalase, which act in anti-radical defense at a stage prior to the formation of the hydroxyl radical. Increased protection has been described against radical damage by enhanced levels of quinone reductase (Murphy et al, 1991). This enzyme plays a role in the detoxification of semiquinone free radicals which can damage cells by reactions independent of the hydroxyl radical. Grunberger and Frenkel (U.S. Patent No. 5,591,773) describe compounds that protect against oxidative stress by decreasing hydrogen peroxide production by polymorphonuclear leukocytes.

While high rates of production of free radicals in cells can be detrimental, there are medical situations where such production is used therapeutically. Anti-cancer radiation therapy involves radiation-induced production of radicals in tumors, and some anticancer drugs act by the production of radicals. However, a detrimental or limiting factor in these therapies is damage to normal tissue by radicals. Approaches that provide selective protection of normal tissues against free radical damage would be of therapeutic benefit, which could be accomplished by administration of agents (compounds) that selectively increase the activity of critical defensive enzymes in normal tissue, such as those of the present invention.

DISCLOSURE OF THE INVENTION

An object of the present invention is to provide agents that protect against the cytotoxic effects of hydrogen peroxide, and which are thereby capable of being

used to treat diseases or repair damage caused by oxygen-containing free radicals produced in cells either from hydrogen peroxide, by other cellular reactions that produce similar oxygen-containing free radicals, or by exogenous agents such as radiation and drugs.

Another object of the invention is to provide a screening method to identify and select agents that confer protection against the cytotoxic effects of hydrogen peroxide and thereby against the cytotoxic effects of cellular free radicals.

Yet another object of the invention is to provide methods of treating or preventing diseases caused by free radicals in humans or animals.

Still another object of the invention is to provide methods of protecting healthy tissue cells from damage by free radicals during the destruction of cancerous cells in cancer therapy.

The inventors set out to identify an enzyme that is critical in protecting against damage caused by the hydroxyl radical and to identify compounds that increase the activity of this enzyme. The identifications involved finding compounds that protect cultured cells against the cytotoxic effects of hydrogen peroxide, which was used as a source of the hydroxyl radical, and then obtaining correlations for increases in protection against hydrogen peroxide with increases in specific activity of enzymes that protect against radical damage. The approach identified glutathione S-transferase as a critical enzyme and led to the identification of compounds that simultaneously increased specific activity of the enzyme and protected against the cytotoxic effects of hydrogen peroxide.

The present invention thus has two crucial aspects: 1) the identification of an enzyme with damage-control activity that is critical in defense against the cytotoxic

effects of hydrogen peroxide, and 2) the identification of agents that increase the activity of this critical enzyme. The action of the agents of the present invention is through elevation of the specific activity of glutathione S-transferase. Glutathione S-transferase occurs as isoforms, some of which have damage-control activities relevant to dealing with hydroxyl radical damage. These activities include 1) the detoxification of phospholipid hydroperoxides, fatty acid hydroperoxides, and alpha-beta unsaturated aldehydes produced by the decomposition of radical-damaged lipids, 2) the repair of radical-damaged DNA and 3) the detoxification of lipid epoxides. The invention relates to increasing the activity of isoforms with appropriate damage-control activities.

In accordance with this invention, compounds have been identified for use as therapeutic agents to prevent, halt or delay the progression of diseases in which pathological damage is caused by free radicals produced either via hydrogen peroxide or by processes independent of hydrogen peroxide, or to minimise damage by such radicals in the other conditions or situations described above. The compounds of the invention act by boosting the level of cellular defenses against free radicals and their products which have been produced either via hydrogen peroxide or by free radicals of the type that can be produced in cells by hydrogen peroxide by physiological processes that do not involve hydrogen peroxide, by increasing enzyme activities that are critically involved in defending against damage caused by oxygen-containing radicals or their products.

Thus, the present invention in one aspect relates to a method of treating diseases or cellular damage caused by oxygen-containing free radicals, or of preventing or minimising such damage, that comprises the step of administering to a patient in need thereof an effective

amount of one or more glutathione S-transferase elevating compounds, preferably selected from the group of compounds consisting of Michael reaction acceptors, halogenated compounds subject to nucleophilic displacement reaction via glutathione S-transferase,1,2-dithiol-3-thiones, triphenols with vicinal hydroxyl groups, nifuroxime and vitamin K-S(II).

It will, therefore, be seen that the present invention broadly relates to a therapy to combat hydrogen peroxide-induced cellular damage, or damage produced by radicals of the type produced in cells from either hydrogen peroxide or other physiological sources, or by free radicals produced by exogenous agents such as radiation and drugs, through treatment with compounds that increase the activity of one or more isoforms of the enzyme glutathione S-transferase involved in defense against, or prevention of, free radical-associated damage. By treatment with specific compounds, there is achieved concurrently an increase in the activity of relevant isoforms of glutathione S-transferase and protection against the toxic effects of hydrogen peroxide, or of radicals of the type that can be produced in cells by hydrogen peroxide, or by radicals of the type produced from hydrogen peroxide by cell process that do not involve hydrogen peroxide, or by radicals produced by exogenous agents such as radiation, drugs or toxins, or by products of the aforementioned radicals.

A feature of the present invention is that glutathione S-transferase can be a critical enzyme in protection against the cytotoxic effects of hydrogen peroxide. Another feature of the invention is the identification of structural classes of compounds that concurrently increase the level of one or more isoforms of glutathione S-transferase that protect against the toxic effects of hydrogen peroxide. Most of these compounds

belong to classes that are known inducers of glutathione S-transferase and of other xenobiotic metabolising enzymes in a number of tissues (Talalay et al, 1988; Spencer et al, 1991; Prestera et al, 1993; Egner et al, 1994). Inducers can be selective for the isoforms they induce (Bogaards et al., 1990; Ketterer et al., 1988; Vos et al., 1988). The invention relates to induction of the isoforms that preferentially catalyze reactions associated with defenses against damage due to free radicals of the type produced via hydrogen peroxide.

In another aspect of the invention, there is provided a method of screening compounds for an ability to induce isoforms of glutathione S-transferase that protect against the cytotoxicity of hydrogen peroxide, comprising: obtaining a culture of mammalian cells with potential to have the specific activity of glutathione S-transferase increased by the addition of exogenous compounds to the culture medium; culturing the cells in the absence and presence of a selected concentration of compounds to be screened and identifying those compounds that increase the specific activity of glutathione S-transferase. Test compounds that at the selected concentration cause an increase in glutathione S-transferase level of at least 10% also cause an increase in resistance to hydrogen peroxide of cells cultured in the presence of the selected concentration of the test compound. The assay for specific activity of glutathione S-transferase uses a particular substrate, 1-chloro-2,4-dinitrobenzene. Isoforms of glutathione S-transferase exhibit appreciable differences in preference for substrates. Use of the substrate 1-chloro-2,4-dinitrobenzene was found empirically to identify compounds that lead to protection against hydrogen peroxide, which was attributed to the preference of isoforms that protect against hydrogen peroxide for the substrate.

An additional feature, and discovery of the invention, is that compound-caused increases in activity of glutathione S-transferase can be related to the level present prior to treatment with the compound. In neuronal and glial cell lines, it is possible for the increases to be greater when the activity of the enzyme prior to exposure to compounds of the invention is lower. The phenomenon indicates that the invention will be most useful in protecting cells against oxygen-containing free radicals when the cells have a relatively low activity of glutathione S-transferase, provided the specific activity of the enzyme can be increased by exogenous inducers. The discovery indicates also that the invention will be useful in providing selective protection of normal tissue in radiation treatment of tumors and in anti-cancer therapy with drugs that act by producing free radicals of the type produced via hydrogen peroxide. Some tumors possess higher levels of glutathione S-transferase than normal adjacent tissues (Siegers et al, 1984: Di Ilio et al, 1985; De Waziers et al, 1991; Peters et al, 1992; Albin et al, 1993; Butler et al, 1994). Administration of compounds of the invention prior to radiation therapy or treatment with radical-producing drugs would then cause a greater increase in activity of glutathione S-transferase in normal tissue relative to tumor tissue, and hence increase the resistance of normal tissue to radiation and drug damage relative to tumor tissue.

Thus, according to another aspect of the invention, there is provided a method of providing selective protection of normal tissue of a patient during radiation treatment of tumors and in anti-cancer therapy with drugs that act by producing free radicals of the type produced via hydrogen peroxide, which method comprises administering to the patient in need thereof an effective amount of one or more compounds that increase the specific

activity of glutathione S-transferase in cells of said patient.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graph which illustrates the critical nature of glutathione S-transferase in protecting against the cytotoxic effects of hydrogen peroxide via correlation analysis between increases in the activity of the enzyme by a compound of the invention, ethacrynic acid, and increases in resistance to hydrogen peroxide;

Figure 2 is a graph which illustrates the critical nature of glutathione S-transferase in protecting against the cytotoxic effects of hydrogen peroxide via correlation analysis between increases in the activity of the enzyme by a compound of the invention, 3-methylene-2-norbornanone, and increases in resistance to hydrogen peroxide; and

Figure 3 is a graph which shows that inducer-caused increases in activity of glutathione S-transferase are greater when the activity of the enzyme in the absence of the inducer is low.

BEST MODES FOR CARRYING OUT THE INVENTION

The invention relates to the use of inducers that activate the enzyme glutathione S-transferase *in vivo* to protect human or animal cells from damage by, or to promote recovery from, oxygen-containing free radicals. The inducers found to be useful in the present invention are known compounds, or compounds that can be produced by known methods, identified by a screening procedure developed by the inventors.

Screening method

The method employed for screening compounds for effectiveness is described in precise detail in Example 1. Basically, the method consists of using a human cell line to identify compounds which, when employed within a specified concentration range, increase the specific

activity of glutathione S-transferase by at least 10% at at least the 95% level of statistical significance. The assay for enzyme activity uses a particular substrate, 1-chloro-2,4-dinitrobenzene. Compounds with the requisite activity increase resistance of the cell line to hydrogen peroxide at the 95% level of statistical significance. Compounds that increase enzyme activity are verified to increase resistance to hydrogen peroxide. Thus, compounds considered to be suitable for the present invention increase both the specific activity of glutathione S-transferase and the resistance to hydrogen peroxide at the 95% level of statistical significance.

The compounds identified by the screening procedure can be predicted to have an effect on the resistance of cells to damage by oxygen-containing free radicals, or the ability of such cells to recover from such damage. Naturally, compounds selected in this way must undergo the usual animal and human trials to select the most effective compounds and (unless the compounds have an appropriate history of human use as drugs or presence in foodstuffs) to ensure an absence of toxicity and adverse side effects. Such testing is a routine part of the development of new drugs and is within the expected skill of a person knowledgeable in the field.

Effective Compounds

Four structural classes of inducers, and two compounds that do not fit into these classes, have been identified by the screening method as being useful according to the present invention. The structural classes consist of the following:

1) Michael reaction acceptors. The Michael reaction consists of the nucleophilic replacement of an active hydrogen in olefinic or acetylenic compounds bearing an electron withdrawing moiety. The acceptors can be substrates for glutathione S-transferase. Electron

withdrawing moieties used successfully include aldehyde, keto, ester, ether and nitro groups. Structures used successfully included the following: alpha-beta unsaturated compounds such as 4-hexene-3-one, trans-2-hexenal, 2-cyclohexene-1-one, 3-methyl-2-cyclohexen-1-one, 5,6-dihydro-2H-pyran-2-one; compounds with two conjugated double bonds such as alpha-ionone and beta-ionone; compounds with methylene groups such as ethacrynic acid, 3-methylene-2-norbornanone, 2-methylene-4-butyrolactone and parthenolide; esters such as diethylfumarate, dimethyl fumarate, ethylenediacrylate, and an ester bearing alkyne group rather than an alkene group, methyl 2-octynoate; an ether such as kahweol; a nitro compound is 1-nitro-1-cyclohexene. Other members in this class consist of compounds that either autoxidize or are metabolised to Michael addition acceptors. Examples include amodiaquine and 2,3-diaminopyridine, which can be oxidized to quinoneimine structures, phenols such as butein, eriodictyol, fisetin, 3-methoxycatechol, myricetin, nordihydroguaiaretic acid, 2',4',6',3,4-pentahydroxy chalcone, quercetin and sulfuretin, which can oxidize to quinones, and butylated hydroxyanisole, which can be metabolized to a quinone.

2) Halogen-containing compounds subject to nucleophilic displacement reaction via glutathione S-transferase. Useful compounds of this class include monochlorobimane {3-chloromethyl-2,5,6-trimethyl-1H,7H-pyrazolo[1,2-a] pyrazole-1,7-dione} and (alpha-bromoisovaleryl)urea {N-(aminocarbonyl)-2-bromo-3-methylbutanamide}.

3) Compounds in the 1,2-dithiol-3-thione class. Useful compounds in this class are anethole trithione {5-(p-methoxyphenyl)-3H-1,2-dithiole-3-thione} and oltipraz {4-methyl-5-(2-pyrazinyl)-1,2-dithiole-3-thione}.

4) Triphenols with vicinyl hydroxyl groups. Useful compounds in this class are propyl gallate, pupurogallin, pyrogallol and 2',3',4'-trihydroxy chalcone.

The additional compounds found to be useful are nifuroxime {5-nitro-2-furancarboxaldehyde} and vitamin K-S(II) {3-[(1,4-dihydro-3-methyl-1,4-dioxo-2-napthalenyl)thio]propanoic acid}.

The effective compounds are considered to include naturally occurring derivatives which, on ingestion, have the potential to be converted into the active compounds. Specific examples are glycosides of butein, eriodictyol, fisetin, myricetin, nordihydroguaiaretic acid, and sulfuretin which can be deglycosylated by intestinal bacteria, and esters of kahweol (e.g. kahweol palmitate), which have potential to be converted to kahweol by human or bacterial enzymes.

While effective compounds have been identified to fall into particular structural classes, more is required than membership in these classes for a compound to be considered as part of the invention. Specifically, the compounds have to successfully pass the screening test for ability to induce glutathione S-transferase and also pass the confirmatory test for ability to protect against the cytotoxicity of hydrogen peroxide. Membership in a compound class does not necessarily make a compound an effective inducer, and prediction of such activities is not obvious. For example, quercetin and fisetin are catechols that are effective inducers and protectors at 10 micromolar, while other catechols such as (+) epicatechin or gossypetin are ineffective at this concentration.

Dosage Forms and Rates

The compounds of the invention may be formulated into sterile pharmaceutical compositions for administration to the mammal to be treated or protected by suitable conventional routes, e.g. orally, parenterally,

application to the skin, etc., using standard pharmaceutically-acceptable ingredients, if required, i.e. adjuvants, excipients, diluents or carriers. Examples of such ingredients include, but are not limited to, water, phosphate-buffered saline solution, emulsifiers, wetting agents, calcium carbonate, chalk, sugars, etc.

The compounds are in each case used in an effective amount, i.e. any amount that will provide noticeable protection or recovery from cellular damage by oxygen-containing free radicals, or protection of healthy cells during cancer treatments.

Effective dosage rates can be worked out from animal tests or clinical trials in the normal manner. Nevertheless, when compounds of the invention are to be injected into humans, a suitable dosage for most or all of the compounds is believed to lie in the range of about 0.1 to 255 mg/kg body weight, and preferably 1 to 10 mg/kg body weight. The most preferred dosage is, of course, the amount which provides the maximum protection against the destructive effects of oxidants present in mammalian cells.

When injectable compositions are desired, the compounds may be formulated, for example, into preparations for injection by dissolving, suspending or emulsifying them into aqueous or non-aqueous solvents, such as vegetable oil, synthetic aliphatic acid glycerides, esters of higher aliphatic acids or propylene glycol; and, if desired, with conventional additives such as solubilizers, isotonic agents, suspending agents, emulsifying agents, stabilizers and preservatives.

The compounds propyl gallate and 5,6-dihydro-2H-pyran-2-one are relatively soluble in water and can be dissolved to form water-based injectable compositions.

Alternatively, if oral dosages are to be prepared, commonly used and pharmaceutically acceptable tabletting

excipients, such as lactose, microcrystalline cellulose, corn starch, stearic acid, or the like, may by used, if desired, to prepare such dosage forms.

The compounds may be formulated into unit dosage form, i.e. discrete units suitable as unitary dosages for the mammal in question, each unit containing a predetermined quantity of a compound of the invention herein mentioned, calculated in a sufficient amount with a pharmaceutically acceptable diluent, carrier or vehicle. The specifications for such unit dosage forms depends on the particular compound employed and the effect to be achieved, etc.

Any necessary adjustments in dose can be readily made to meet the severity of the disorder encountered and adjusted accordingly by the skilled practitioner.

It will be apparent that the compounds identified as suitable for use in the invention can be used in conventional ways for the manufacture of pharmaceutical compositions in unit dosage form suitable for the treatment of the diseases mentioned above. Such methods of manufacture are well known to persons skilled in the art and need not be given here.

Additional Testing

As will be apparent to a person skilled in the art, the compounds used in the present invention, at the appropriate dosage rates, must be screened for toxicity and unwanted side effects in humans or animals before being released onto the market. Non-toxic compounds having only acceptable side effects or no side effects at all should normally be selected (although compounds used for cancer treatment are often somewhat toxic and have unwanted side effects since the criticality of the disease often justifies harsh treatments). To the extent that compounds selected for the present invention have not already undergone appropriate testing for toxicity and

side effects, and if the compounds do not already exist in foodstuffs (suggesting their harmless characteristics), the appropriate additional testing will be carried out before commercial products are made available. The nature of such additional tests are well known to persons skilled in the art and need not be give here.

Further preferred embodiments, advantages and features of the invention are illustrated by the following non-limiting Examples.

For the following Examples, all of the compounds used are commercially available, are known in the literature or can be produced by common methods of synthesis that would be apparent to any person skilled in the art without undue experimentation.

Example 1

This Example describes the procedure used to screen for compounds that protect against the cytotoxic effects of hydrogen peroxide by the induction of the enzyme glutathione S-transferase. This Example also serves to describe the methods used in other Examples to show that glutathione S-transferase is a critical enzyme in protecting against the cytotoxicity of hydrogen peroxide and to demonstrate other features of the invention.

The screening procedure involves two separate protocols. One protocol consists of an assay to quantify compound-caused increases in the specific activity of glutathione S-transferase in cell extracts of cultured cells by at least 10% when measured using 1-chloro-2,4-dinitrobenzene as substrate. The other protocol consists of measuring the increase in resistance of the cultured cells to hydrogen peroxide caused by treatment with compounds that increase the specific activity of glutathione S-transferase. Compounds that increased the specific activity of glutathione S-transferase when assayed as described increased resistance to hydrogen

peroxide, but both protocols were always used to identify compounds.

Compounds that increased both protection against hydrogen peroxide and the specific activity of glutathione S-transferase at the 95% level of statistical significance at compound concentrations at or below a specified limit were considered to be compounds of the invention. The concentration limit was used to select for the more potent compounds, and except for two compounds, was 10 micromolar or less.

The specific activity of glutathione S-transferase was measured using 1-chloro-2,4-dinitrobenzene as a substrate (Habig et al, 1974) in cell-free homogenates prepared from washed, trypsinised cells that had been cultured on the surface of 25 cm^2 tissue culture flasks in the presence or absence of an inducer for forty-eight hours. The homogenates were stored at -80°C prior to use. The assay was carried out using either 1 ml volumes of cell extracts in spectrophotometer cuvettes or 200 microliter volumes in ninety-six well plates. Each determination was carried out at least in triplicate.

The effect of compounds that increase glutathione S-transferase activity on protection against hydrogen peroxide was evaluated by measuring the IC_{50} for hydrogen peroxide. The IC_{50} is the concentration of hydrogen peroxide that causes a 50% percent decrease in a measure of cell viability. Compounds that increased the IC_{50} increase the resistance of cells to the cytotoxic effects of hydrogen peroxide. The assay employed a tetrazolium salt to measure the IC_{50}. The tetrazolium salt used was 3-[4,5-dimethylthiazol-2-yl]-2,5- diphenyltetrazolium bromide (MTT). This compound is reduced by cellular enzymes, and the extent of reduction is taken as a measure of cell viability (Hansen et al, 1989). The assay was carried out in 96 well plates. Each well was inoculated

with the same number of cells, approximately 1000, and the cultures grown for forty-eight hours with or without test compound as required. The culture medium was then removed and the cultures subjected to a series of concentrations of hydrogen peroxide for 2 hours, after which the hydrogen peroxide was removed and the cells treated with the tetrazolium salt. The extent of reduction of the tetrazolium salt was quantitated spectrophotometrically. The results were plotted as the percent of control absorbance, the control absorbance being the value given by cells untreated with hydrogen peroxide. The concentration of hydrogen peroxide that gave a 50% decrease in absorbance was taken to represent the IC_{50}. Determinations for each concentration of hydrogen peroxide were carried out in quadruplicate.

The cell lines used are commercially available and were obtained from the American Type Culture Collections (ATCC), of Rockville, Maryland, USA. They were the neuronal cell lines IMR-32 (ATCC Number CCL-127), Neuro 2a (ATCC Number CCL-131), and the glial cell lines U-87 MG (ATCC Number HTB-14), U-373 MG (ATCC Number HTB-17) and C_6 (ATCC Number CCL-107). They were stored in the frozen state in liquid nitrogen.

Working cultures of the cell lines were grown on the surface of plastic flasks in Modified Eagle's Medium supplemented with 10% fetal calf serum, 1 millimolar sodium pyruvate, penicillin G and streptomycin at 37°C in an atmosphere of 8% carbon dioxide and 92% air. The working cultures were transferred every four days, and new working cultures were established from frozen stocks after ten transfers. The working cultures were used to provide cells for the glutathione S-transferase and hydrogen peroxide cytotoxicity assays. The culture medium and culture conditions used for the assays was the same as that for the working cultures. The test compounds were

added to the culture medium in the form of concentrated solutions in ethanol or dimethyl sulfoxide. The final concentration of solvent in the culture medium was 0.1%, which did not affect the results. Trypsinisation of cells for removal from the plastic surface and dispersion in medium was carried out as described elsewhere (Schneider et al, 1993).

Example 2

This Example shows that glutathione S-transferase can be a critical enzyme in defense against the cytotoxic effects of hydrogen peroxide.

A criterion for a critical enzyme in defense against hydrogen peroxide cytotoxicity is that the IC_{50} for hydrogen peroxide should increase when specific activity of the critical enzyme increases. The existence of this relation for glutathione S-transferase was demonstrated by assessment of the correlation between increases in specific activity of the enzyme with increases in IC_{50} when specific activity was varied by exposing cultured cells to a range of concentration of compounds of the invention. When the concentration of ethacrynic acid (as shown in Figure 1) or 3-methylene-2-norbornanone (as shown in Figure 2) were increased in cultures of the cell line IMR-32, the specific activity of glutathione S-transferase and the IC_{50} both increased. In each case, the increase in glutathione S-transferase was effected by culturing the cells over a range of concentrations of ethacrynic acid or 3-methylene-2-norbornanone. In addition, the coefficients of determination r^2, a measure of correlation between increases in specific activity of the enzyme and the IC_{50}, were close to 1; 0.931 ($p=0.0001$) with ethacrynic acid, and 0.959 ($p=0.0001$) with 3-methylene-2-norbornanone. Values of r^2 close to 1 indicate a high degree of correlation between the specific activity of glutathione S-transferase and IC_{50}. The lines shown were obtained by

linear regression and are shown to aid in visualization of the data.

Specifically, Figure 1 shows the correlation between the percent increase in IC_{50} and specific activity of glutathione S-transferase (GST) in the cell line IMR-32. The changes in IC_{50} and specific activity of the enzyme were effected by culturing cells from the same parent flask for forty-eight hours in the presence of a range of concentrations of ethacrynic acid. Each of the points shown was obtained using a different concentration. The point on the extreme left was obtained with the lowest concentration used, 0.5 micromolar, and the point at the extreme right with the highest concentration, 25 micromolar. Figure 2 shows the correlation between the percent increase in IC_{50} and specific activity of glutathione S-transferase in IMR-32 for a range of concentrations of 3-methylene-2-norbornanone. The point at the extreme left was obtained with the lowest concentration used, 0.05 micromolar, and the point at the extreme right with the highest concentration used, 2 micromolar.

Example 3

This Example shows that each of the compounds of the invention concurrently increases the specific activity of glutathione S-transferase and the IC_{50} for hydrogen peroxide in the cell line IMR-32.

Each of the compounds of the invention was tested at concentrations of 25 micromolar or less for concurrent increases in the specific activity of glutathione S-transferase level and IC_{50} for hydrogen peroxide. Table 1 shows the concurrent increases, expressed as percent increase, for single concentrations of each of the compounds.

Table 1

Increases in glutathione S-transferase and IC_{50} caused by selected compounds. Increases reported as means ± standard error (number of determinations)

Compound type	Compound	Concentration (micromolar)	GST (percent increase)	IC_{50} (percent increase)
Michael addition	Amodiaquine	10	18.9 ± 3.93 (3)	279 ± 64 (3)
	Butein	10	86.5 ± 18.7 (3)	106 ± 46 (5)
	Butylated hydroxyanisole	10	25.4 ± 1.44 (3)	315 ± 17 (3)
	2-Cyclohexen-1-one	10	102 ± 20 (4)	56.3 ± 6.9 (3)
	2,3-Diamino pyridine	10	26.2 ± 5.12 (3)	75.7 ± 20.1 (4)
	Diethyl fumarate	10	171 ± 21 (4)	423 ± 62 (3)
	Dimethyl fumarate	10	167 ± 21 (4)	293 ± 8 (3)
	5,6-Dihydro-2H-pyran-2-one	10	173 ± 31 (4)	416 ± 90 (3)
	Eriodictyol	10	92.2 ± 10.6 (4)	187 ± 47 (4)
	Ethacrynic acid	25	259 ± 33 (5)	204 ± 38 (3)
	Ethylenediacrylate	10	136 ± 17 (4)	61.7 ± 0.5 (2)
	Fisetin	10	114 ± 1.2 (3)	361 ± 45 (2)
	4-Hexene-3-one	10	110 ± 7.8 (4)	67.0 ± 12.7 (3)
	trans-2-hexenal	10	19.0 ± 6.2 (4)	23.6 ± 1.9 (3)
	alpha-ionone	10	31.9 ± 5.6 (3)	29.7 ± 17.7 (3)
	beta-ionone	10	74.8 ± 17.6 (3)	36.2 ± 10.3 (4)

	Kahweol	10	24.2 ± 3.7 (4)	39.9 ± 10.6 (3)
	3-Methoxycatechol	10	129 ± 26 (2)	123 ± 44 (3)
	2-Methylene-4-butyrolactone	2	63.1 ± 15.8 (3)	59.0 ± 10.1 (3)
	3-Methyl-2-cyclohexen-1-one	10	22.4 ± 4.6 (2)	55.3 ± 6.8 (3)
	3-Methylene-2-norbornanone	2	239 ± 40 (4)	172 ± 51 (4)
	Methyl-2-octynoate	10	226 ± 21 (3)	137 ± 18 (3)
	Myricetin	10	117 ± 4 (2)	56.8 ± 20.5 (3)
	1-Nitro-1-cyclohexene	2	181 ± 10(4)	238 ± 53 (3)
	Nordihydro-guaiaretic acid	10	89.5 ± 29.9 (3)	166 ± 59 (3)
	Parthenolide	2	107 ± 8 (4)	54.5 ± 15 (4)
	2',4',6',3,4-Penta-hydroxychalcone	10	66.7 ± 8.3 (3)	96.7 ± 24 (4)
	Quercetin	10	137 ± 13 (3)	245 ± 64 (2)
	Sulfuretin	10	79.4 ± 3.2 (3)	87.1 ± 39.5 (2)
Halogen compounds	alpha Bromo-isovaleryl urea	25	76.5 ± 3.0 (3)	79.1 ± 6.2 (4)
	Monochlorobimane	10	133 ± 10 (5)	123 ± 11 (4)
Dithiolthiones	Anethole trithione	10	49.2 ± 3.5 (4)	167 ± 28 (4)
	Oltipraz	10	13.8 ± 2.1 (4)	200 ± 39 (4)
Triphenols	Propyl gallate	10	134 ± 38 (3)	114 ± 19 (3)
	Purpurogallin	10	45.8 ± 9.3 (3)	24.7 ± 4.7 (3)
	Pyrogallol	10	149 ± 19 (3)	182 ± 64 (3)

	2',3',4'-Trihydroxy-chalcone	10	112 ± 21 (3)	387 ± 138 (2)
Other compounds	Nifuroxime	10	172 ± 11 (4)	292 ± 19 (3)
	Vitamin K-S(II)	10	75.8 ± 6.5 (3)	200 ± 74 (3)

All of the compounds in the Table increased both protection against hydrogen peroxide and the specific activity of glutathione S-transferase at the 95% confidence level as determined in multiple determinations using different batches of cells. The statistical significance of differences between IC_{50} values and glutathione S-transferase activities in the presence and absence of compounds of the invention was determined using the one-tailed paired t-test.

Example 4

This Example shows that the extent to which glutathione S-transferase can be increased by an inducer is greater in cell lines which have lower levels of activity prior to exposure to the inducer. The phenomenon indicates that a preferred application of the invention in preventing free radical damage is in cells in which the enzyme is inducible and present at low levels, and that the invention can be used to provide selective protection of normal tissue in radiation or drug therapy directed against cancers with high specific activities of the enzyme.

The Example measured the extent to which a compound of the invention (ethacrynic acid) increases the specific activity of glutathione S-transferase in cell lines with similar or related functions (neuronal and glial) which, in the absence of treatment with an inducer, differ in the specific activity for the enzyme. Cell lines that had

relatively low activities of glutathione S-transferase in the absence of inducer manifested greater inducer-caused increases in activity of the enzyme (Figure 3). Notably, cell lines with the highest specific activities of glutathione S-transferase showed very little if any increase in enzyme activity.

Specifically, Figure 3 shows the dependence of the inducability of glutathione S-transferase, referred to as GST in Figure 3, with basal activity of the enzyme. The inducer was ethacrynic acid at 25 micromolar. Linear regression on appropriately transformed vertical and horizontal axes indicated that the downward trend of percent increase in glutathione S-transferase activity as basal activity increased was significant; the slope was nonzero with $p = 0.0098$. Each point represents a different cell line, which differ in glutathione S-transferase activity in the absence of inducer (A = IMR-32, B = C_6, C = Neuro 2a, D = U-87-MG and E = U-373 MG). The points represent the mean of values obtained in independent determinations. The error bars represent standard deviations. The number of independent determinations to determine the basal activity of glutathione S-transferase was five or six for all cell lines except for IMR-32 where it was three. The corresponding numbers for the percent increase in activity was four for all cell lines.

Example 5

This Example shows that compounds of the invention can be used prophylactically to protect against tissue damage caused by ischemia-reperfusion, and also against damage to brain neurons that occurs as the result of the ischemia-reperfusion associated with stroke. Because ischemia-reperfusion is associated with free radical damage, the Example also shows that the compounds can be used to alleviate such damage prophylactically in other

conditions where oxygen-containing free radicals are pathological.

The Example employed the four-vessel occlusion model of stroke in rats, which consists of subjecting the brain of rats to ischemia-reperfusion by stopping blood flow to the brain for a defined period of time and then allowing reperfusion of blood to the brain to occur. The model was chosen because it permits simultaneous demonstration of the effectiveness of compounds of the invention in preventing damage caused by ischemia-reperfusion in a tissue, as well as in preventing damage in a model of a particular disease, namely stroke. The model was also chosen because of the similarity between the method used to cause free radical damage in the *in vitro* selection procedure to identify compounds of the invention, and the process thought to cause free radical damage during ischemia-reperfusion and other diseases or conditions in which free radicals cause pathological damage. Specifically, on the one hand, hydrogen peroxide was used as the source of the hydroxyl radical in the *in vitro* selection procedure to identify compounds of the invention. On the other hand, elevated levels of hydrogen peroxide are produced by ischemia-reperfusion in the brain (Hyslop et al., 1995; Lei et al., 1997a; Lei et al., 1997b; Simonson et al., 1993) and in other tissues of animals and humans (Ko et al., 1991; Mathru et al., 1996; Slezak et al., 1995; Smielecki et al., 1996). In addition, hydrogen peroxide is produced in other tissues by conditions other than ischemia-reperfusion (Dekhuijzen et al., 1996; Dohlman et al., 1993; Gonzalez-Flecha et al., 1993; Ketizmann et al., 1993; Winrow et al., 1993). Moreover, the hydroxyl radical is produced by ischemia-reperfusion in the brain (Hall et al., 1993; Kil et al., 1996; Morimoto et al., 1996; Piantadosi and Zhang, 1996) and other tissues of animals (Fisher et al., 1993;

Kadkhodaee et al., 1995; Kunz et al., 1991; Muller et al., 1997; O'Neill et al., 1996; Ophir et al., 1993; Premaratne et al., 1994; Rose et al., 1994; Sun et al., 1993; Timoshin et al., 1994).

The procedure used to cause ischemia-reperfusion in the model is described in the scientific literature (Li and Buchan, 1993). Male Wistar rats in the 200-260 gram range were employed. Briefly, under inhalation anesthesia the two vertebral arteries were permanently occluded, and ischemia initiated by clamping off the internal carotid arteries. The ischemic period used was ten minutes. Body temperature was maintained at 37.0°C using a heating pad, and tympanic temperature was maintained at 37.3°C using a heating lamp focussed on the head and a miniature probe inserted into the auditory canal. Animals that were unresponsive and had dilated pupils were used in the study while animals that exhibited a seizure or died were excluded.

Neuronal damage caused by the ischemia-reperfusion episode was assessed five days after the episode by the extent of damage to the CA1 pyramidal hippocampal neurons, which are known to be sensitive to damage caused by ischemia-reperfusion. The extent of neuronal damage was quantitated histologically by determining the number of normal-appearing and injured pyramidal CA1 neurons, averaging the results for the two cerebral hemispheres of each animal, and calculating the percentage of damaged neurons. Counting of the normal and damaged neurons was done in a blinded manner. Comparisons between the treated and untreated groups were made using the Mann-Whitney procedure, with statistical significance being accepted when p was less than 0.05. In brief, preparation of the histological specimens involved the following steps: perfusion of the brain of the animals with 4% buffered formalin, removal of the brain from the animals, embedding

the brain in paraffin, preparation of 7 μm thick sections through the dorsal hippocampus and staining of the sections with haematoxylin and eosin.

The objective of prophylactic treatment is to protect tissue from free radical damage when ischemia-reperfusion is anticipated, by boosting the level of a critical enzyme in defense against free radical damage prior to the occurrence of the ischemia-reperfusion episode. Examples of expected damage are when ischemia-reperfusion in brain is anticipated in patients at risk for stroke, in tissues of patients about to undergo surgical procedures causing ischemia-reperfusion, or when there is the possibility that a blood clot in one location can move to another location and block blood flow at this other location. In the Example each treated animal received two doses of the test compound dissolved or suspended in vehicle: one dose at forty-eight hours and another twenty-four hours prior to ischemia-reperfusion. Control animals were treated identically in that they underwent the ischemia-reperfusion procedure, but were treated with the vehicle alone. The drugs were administered intraperitoneally.

Table 2 shows that compounds of the invention prophylactically decrease damage to pyramidal CA1 hippocampal neurons in an ischemia-reperfusion model of stroke, in that the percent damage was smaller in treated animals.

Table 2

Compound	Concentration (mg/kg)	Route	Percent damage mean ± std. err (N)		p
			control	treated	
alpha Bromo-isovaleryl urea	50[2]	i.p.	82.0 ± 2.2 (17)	71.6 ± 4.9 (5)	.0172
Diethylfumarate	255[1]	i.p.	82.8 ± 4.1 (16)	58.7 ± 8.0 (13)	.0101
Methyl-octynoate	75[3]	i.p.	84.6 ± 1.6 (5)	65.9 ± 5.6 (5)	.0079
	100[1]		82.8 ± 4.1 (16)	69.8 ± 6.0 (12)	.0027
	150[1]		82.8 ± 4.1 (16)	71.9 ± 2.3 (11)	.0004
Monochloro-bimane	12.5[2]	i.p.	82.0 ± 2.2 (17)	70.7 ± 6.7 (5)	.0301
Quercetin	32[2]	i.p.	82.0 ± 2.2 (17)	66.3 ± 6.4 (12)	.0127
Vitamin K-S(II)	12.5[2]	i.p.	82.0 ± 2.2 (17)	74.7 ± 2.8 (15)	.0271

[1]Vehicle = canola oil(commercially available material obtained from local supermarket); injection volume was 0.5 ml per 200 gram rat.

[2]Vehicle = 0.5% carboxymethyl cellulose (obtained from the Sigma Chemical Company, St. Louis, MO, viscosity 3000-6000 centipoise in a 1% aqueous solution) in isotonic saline; injection volume was 0.5 ml per 200 gram rat.

[3]Vehicle = Emulsion in 1% Cremophor™ EL in isotonic saline; injection volume was 0.5 ml per 200 gram rat.

Example 6

This Example shows that compounds of the invention protect against tissue damage caused by ischemia-reperfusion, and against damage to brain neurons caused by ischemia-reperfusion in an animal model of stroke, when administered after the ischemia-reperfusion episode. Ischemia-reperfusion in brain initiates pathological free radical production and damage to neurons, hence the Example demonstrates that the compounds protect after initiation of the pathological free radical damage in stroke. Because ischemia-reperfusion is also associated with free radical damage more generally, the Example also shows that the compounds can be used to alleviate such

damage when administered after free radical generation has been initiated in other conditions where oxygen-containing free radicals are pathological.

The objective of treatment after the ischemia-reperfusion episode is to increase defenses against free radical damage after the damage process has been initiated. The model employed was identical to that of Example 5 with the difference that the animals were treated with compounds of the invention after reperfusion. Each treated animal received three doses of the drug dissolved or suspended in the vehicle, one dose immediately after the stroke and then at twenty-four and at forty-eight hours later. Control animals were treated identically, but received vehicle only.

Table 3 shows that compounds of the invention decrease damage to pyramidal CA1 hippocampal neurons when administered after ischemia-reperfusion in that the percentage of damage was smaller in the treated animals.

Table 3

Compound	Concentration (mg/kg)	Route	Percent damage mean ± std. err (N)		p
			control	treated	
Diethylfumarate	255[1]	i.p.	81.0 ± 3.9 (15)	66.1 ± 5.1 (16)	.0104
5,6-Dihydro-2H-pyran-2-one	5[2]	i.p.	85.4 ± 1.5 (14)	74.1 ± 4.1 (15)	.0019
	7.5[2]	i.p.	85.4 ± 1.5 (14)	67.7 ± 8.3 (8)	.0035
beta Ionone	150[1]	i.p.	81.0 ± 3.9 (15)	69.3 ± 6.9 (13)	.0311

[1]Vehicle = canola oil; injection volume was 0.5 ml per 200 gram rat.
[2]Vehicle = isotonic saline; injection volume was 0.5 ml per 200 gram rat.

Example 5 above shows prophylactic protection and Example 6 shows acute protection in an animal model. The model is essentially one for ischemia-reperfusion damage

in a tissue, and it is also a model for stroke because such damage occurs in the brain in stroke. Therefore, the data can be used to show the effectiveness of the compounds against ischemia-reperfusion damage in general and stroke in particular. However, the model exemplifies more than protection against ischemia-reperfusion. Because ischemia-reperfusion causes free radical damage, the data also shows that the compounds can be used to treat free radical diseases that do not involve ischemia-reperfusion per se.

The invention being thus described, it will be apparent that the same may be varied in many ways. Such variations are not to be regarded as a departure from the spirit and scope of the invention, and all such modifications as would be apparent to a person skilled in the art are intended to be included within the scope of the present invention.

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The listing of these references should not be taken to imply that the references are considered to be prior art against the invention defined in this application.

CLAIMS:

1. A method of treating diseases or cellular damage in humans or animals caused by oxygen-containing free radicals, or of prophylactically preventing or minimising such damage, or of selectively increasing the resistance of normal tissue relative to cancer tissue to free radical damage, which comprises administering to a patient in need thereof an effective amount of one or more compounds that increase the specific activity of glutathione S-transferase in cells of said patient.

2. A method according to claim 1, wherein said one or more compounds is selected from the group consisting of nifuroxime and vitamin K-S(II), Michael reaction acceptors or compounds that become such acceptors on oxidation or metabolism, halogen compounds subject to nucleophilic displacement reaction via glutathione S-transferase, 1,2-dithiol-3-thiones, and triphenols with vicinal hydroxyl groups.

3. A method according to claim 2, wherein the Michael reaction acceptors are olefinic or acetylenic compounds which contain an electron withdrawing moiety selected from the group consisting of aldehyde, keto, ester, ether and nitro groups as electrophilic groups.

4. A method according to claim 3, wherein said compound is selected from the group consisting of 4-hexene-3-one, trans-2-hexenal, 2-cyclohexene-1-one, 3-methyl-2-cyclohexen-1-one, 5,6-dihydro-2H-pyran-2-one, alpha-ionone, beta-ionone, ethacrynic acid, 3-methylene-2-norbornanone, 2-methylene-4-butyrolactone, diethyl fumarate, dimethyl fumarate, ethylenediacrylate, methyl 2-

octynoate, kahweol, parthenolide, and 1-nitro-1-cyclohexene.

5. A method according to claim 2, wherein the compounds which become Michael reaction acceptors on oxidation or metabolism are selected from the group amodiaquine, butein, butylated hydroxyanisole, 2,3-diaminopyridine, eriodictyol, fisetin, 3-methoxycatechol, myricetin, nordihydroguiaretic acid, 2',4',6',3,4-pentahydroxychalcone, quercetin, and sulfuretin.

6. A method according to claim 2 wherein the compounds subject to nucleophilic displacement reaction via glutathione S-transferase are halogenated hydrocarbons.

7. A method according to claim 6 wherein the halogenated hydrocarbons are selected from the group consisting of monochlorobimane and (alpha-bromoisovaleryl)urea.

8. A method according to claim 2, wherein the compounds are 1,2-dithiol-3-thiones.

9. A method according to claim 8, wherein the compounds are selected from the group consisting of anethole trithione and oltipraz.

10. A method according to claim 2, wherein the compounds are triphenols with vicinal hydroxyl groups.

11. A method according to claim 10, wherein the compounds are selected from the group consisting of propyl gallate, purpurogallin, pyrogallol, and 2',3',4'-trihydroxychalcone.

12. A method according to claim 2, wherein the compounds are selected from nifuroxime and vitamin K-S(II).

13. A method of screening and identifying compounds for an ability to induce isoforms of glutathione S-transferase that protect against free radical damage of the type produced by exposure to hydrogen peroxide, comprising:

obtaining a culture of mammalian cells with the potential to have the specific activity of glutathione S-transferase increased by induction with an exogenous compound;

culturing the cells in the absence and presence of test compounds at a predetermined concentration;

identifying compounds that increase the specific activity of glutathione S-transferase at a statistically significant level;

verifying that the compounds identified protect against the cytotoxic effects of hydrogen peroxide by subjecting the cells to increasing concentrations of hydrogen peroxide in both the presence and absence of the predetermined concentrations of the compound identified, and confirming that, at a statistically significant level, the compounds increase the concentration of hydrogen peroxide causing a specific level of cell viability compared to the cells in the absence of such compounds.

14. A method according to claim 13, wherein said specific level of cell viability is 50% (IC_{50}).

15. A method according to claim 13, wherein a tetrazolium salt is used to measure said specific level of cell viability.

16. A method according to claim 13, wherein the specific activity of glutathione S-transferase is measured using 1-chloro-2,4-dinitrobenzene as a substrate.

17. A method of treating diseases caused by ischemia-reperfusion in humans or animals or cellular damage caused by ischemia-reperfusion, comprising administering to a patient in need thereof an effective amount of one or more compounds that increase the specific activity of isoforms of glutathione S-transferase that protect against free radical damage.

18. A method according to claim 17, wherein said one or more compounds is selected from the group consisting of nifuroxime and vitamin K-S(II), Michael reaction acceptors or compounds that become such acceptors on oxidation or metabolism, halogen compounds subject to nucleophilic displacement reaction via glutathione S-transferase, 1,2-dithiol-3-thiones, and triphenols with vicinal hydroxyl groups.

19. A method according to claim 18, wherein the Michael reaction acceptors are olefinic or acetylenic compounds which contain an electron withdrawing moiety selected from the group consisting of aldehyde, keto, ester, ether and nitro groups as electrophilic groups.

20. A method according to claim 19, wherein said compound is selected from the group consisting of 4-hexene-3-one, trans-2-hexenal, 2-cyclohexene-1-one, 3-methyl-2-cyclohexen-1-one, 5,6-dihydro-2H-pyran-2-one, alpha-ionone, beta-ionone, ethacrynic acid, 3-methylene-2-norbornanone, 2-methylene-4-butyrolactone, diethyl fumarate, dimethyl fumarate, ethylenediacrylate, methyl 2-

octynoate, kahweol, parthenolide, and 1-nitro-1-cyclohexene.

21. A method according to claim 18, wherein the compounds which become Michael reaction acceptors on oxidation or metabolism are selected from the group amodiaquine, butein, butylated hydroxyanisole, 2,3-diaminopyridine, eriodictyol, fisetin, 3-methoxycatechol, myricetin, nordihydroguiaretic acid, 2',4',6',3,4-pentahydroxychalcone, quercetin, and sulfuretin

22. A method according to claim 18 wherein the compounds subject to nucleophilic displacement reaction via glutathione S-transferase are halogenated hydrocarbons.

23. A method according to claim 22 wherein the halogenated hydrocarbons are selected from the group consisting of monochlorobimane and (alpha-bromoisovaleryl)urea.

24. A method according to claim 18, wherein the compounds are 1,2-dithiol-3-thiones.

25. A method according to claim 24 wherein the compounds are selected from the group consisting of anethole trithione and oltipraz.

26. A method according to claim 18, wherein the compounds are triphenols with vicinal hydroxyl groups.

27. A method according to claim 26, wherein the compounds are selected from the group consisting of propyl gallate, purpurogallin, pyrogallol, and 2',3',4'-trihydroxychalcone.

28. A method according to claim 18, wherein the compounds are selected from nifuroxime and vitamin K-S(II).

29. A method according to claim 17 where one or more of the compounds is selected from the group consisting of diethylfumarate, 5,6-dihydro-2H-pyrane-2-one, beta-ionone, (alpha-bromoisovaleryl)urea, methyl octynoate, monochlorobimane, quercetin and vitamin K-S(II).

30. A method of treating stroke by administration to a patient in need thereof an effective amount of one or more compounds that increase the specific activity of isoforms of glutathione S-transferase that protect against free radical damage.

31. A method according to claim 30, wherein said one or more compounds is selected from the group consisting of nifuroxime and vitamin K-S(II), Michael reaction acceptors or compounds that become such acceptors on oxidation or metabolism, halogen compounds subject to nucleophilic displacement reaction via glutathione S-transferase, 1,2-dithiol-3-thiones, and triphenols with vicinal hydroxyl groups.

32. A method according to claim 31, wherein the Michael reaction acceptors are olefinic or acetylenic compounds which contain an electron withdrawing moiety selected from the group consisting of aldehyde, keto, ester, ether and nitro groups as electrophilic groups.

33. A method according to claim 32, wherein said compound is selected from the group consisting of 4-hexene-3-one, trans-2-hexenal, 2-cyclohexene-1-one, 3-methyl-2-

cyclohexen-1-one, 5,6-dihydro-2H-pyran-2-one, alpha-ionone, beta-ionone, ethacrynic acid, 3-methylene-2-norbornanone, 2-methylene-4-butyrolactone, diethyl fumarate, dimethyl fumarate, ethylenediacrylate, methyl 2-octynoate, kahweol, parthenolide, and 1-nitro-1-cyclohexene.

34. A method according to claim 31, wherein the compounds which become Michael reaction acceptors on oxidation or metabolism are selected from the group amodiaquine, butein, butylated hydroxyanisole, 2,3-diaminopyridine, eriodictyol, fisetin, 3-methoxycatechol, myricetin, nordihydroguiaretic acid, 2',4',6',3,4-pentahydroxychalcone, quercetin, and sulfuretin

35. A method according to claim 31 wherein the compounds subject to nucleophilic displacement reaction via glutathione S-transferase are halogenated hydrocarbons.

36. A method according to claim 35 wherein the halogenated hydrocarbons are selected from the group consisting of monochlorobimane and (alpha-bromoisovaleryl)urea.

37. A method according to claim 31, wherein the compounds are 1,2-dithiol-3-thiones.

38. A method according to claim 37 wherein the compounds are selected from the group consisting of anethole trithione and oltipraz.

39. A method according to claim 31, wherein the compounds are triphenols with vicinal hydroxyl groups.

40. A method according to claim 39, wherein the compounds are selected from the group consisting of propyl gallate, purpurogallin, pyrogallol, and 2',3',4'-trihydroxychalcone.

41. A method according to claim 31, wherein the compounds are selected from nifuroxime and vitamin K-S(II).

42. A method according to claim 30, wherein one or more of the compounds is selected from the group consisting of diethyl fumarate, 5,6-dihydro-2H-pyran-2-one and beta-ionone.

43. A method of treatment to prevent free radical damage when ischemia-reperfusion is expected or might occur, comprising administration to an individual in need thereof an effective amount of one or more compounds that increase the specific activity of isoforms of glutathione S-transferase that protect against free radical damage.

44. A method according to claim 43, wherein said one or more compounds is selected from the group consisting of nifuroxime and vitamin K-S(II), Michael reaction acceptors or compounds that become such acceptors on oxidation or metabolism, halogen compounds subject to nucleophilic displacement reaction via glutathione S-transferase, 1,2-dithiol-3-thiones, and triphenols with vicinal hydroxyl groups.

45. A method according to claim 44, wherein the Michael reaction acceptors are olefinic or acetylenic compounds

which contain an electron withdrawing moiety selected from the group consisting of aldehyde, keto, ester, ether and nitro groups as electrophilic groups.

46. A method according to claim 45, wherein said compound is selected from the group consisting of 4-hexene-3-one, trans-2-hexenal, 2-cyclohexene-1-one, 3-methyl-2-cyclohexen-1-one, 5,6-dihydro-2H-pyran-2-one, alpha-ionone, beta-ionone, ethacrynic acid, 3-methylene-2-norbornanone, 2-methylene-4-butyrolactone, diethyl fumarate, dimethyl fumarate, ethylenediacrylate, methyl 2-octynoate, kahweol, parthenolide, and 1-nitro-1-cyclohexene.

47. A method according to claim 44, wherein the compounds which become Michael reaction acceptors on oxidation or metabolism are selected from the group amodiaquine, butein, butylated hydroxyanisole, 2,3-diaminopyridine, eriodictyol, fisetin, 3-methoxycatechol, myricetin, nordihydroguiaretic acid, 2',4',6',3,4-pentahydroxychalcone, quercetin, and sulfuretin

48. A method according to claim 44 wherein the compounds subject to nucleophilic displacement reaction via glutathione S-transferase are halogenated hydrocarbons.

49. A method according to claim 48 wherein the halogenated hydrocarbons are selected from the group consisting of monochlorobimane and (alpha-bromoisovaleryl)urea.

50. A method according to claim 44, wherein the compounds are 1,2-dithiol-3-thiones.

51. A method according to claim 50, wherein the compounds are selected from the group consisting of anethole trithione (5-(p-methoxyphenyl)-3H-1,2-dithiole-3-thione) and oltipraz (5-(2-pyranzinyl)-4-methyl-1,2-dithiol-3-thione).

52. A method according to claim 44, wherein the compounds are triphenols with vicinal hydroxyl groups.

53. A method according to claim 52, wherein the compounds are selected from the group consisting of propyl gallate, purpurogallin, pyrogallol, and 2',3',4'-trihydroxychalcone.

54. A method according to claim 44, wherein the compounds are selected from nifuroxime and vitamin K-S(II).

55. A method according to claim 43 where one or more of the compounds is selected from the group consisting of diethylfumarate, alpha-bromoisovalerylurea, methyl octynoate, monochlorobimane, quercetin and vitamin K-S(II).

56. A method of providing selective protection of normal tissue of a patient during radiation treatment of tumors and in anti-cancer therapy with drugs that act by producing free radicals of the type produced via hydrogen peroxide, which method comprises administering to the patient in need thereof an effective amount of one or more compounds that increase the specific activity of isoforms

of glutathione S-transferase that defend against free radical damage in cells of said patient.

57. A pharmaceutical composition in dosage form suitable for oral or parenteral administration for treating or preventing diseases in humans and animals caused by oxygen-containing free radicals, which comprises a compound that increases the specific activity of isoforms of glutathione S-transferase that protect against free radical damage in cells of said human or animal in admixture with a suitable adjuvant, excipient, diluent or carrier.

58. Use of a compound that increases the specific activity of isoforms of glutathione S-transferase that defend against free radical damage in human or animal cells for the treatment or prophylaxis of diseases caused by oxygen-containing free radicals, or to protect healthy cells during cancer treatments that generate oxygen-containing free radicals.

59. Use of a compound that increases the specific activity of isoforms of glutathione S-transferase that protect against free radical damage in human or animal cells for the manufacture of a composition in unit dosage form for the treatment or prophylaxis of diseases caused by oxygen-containing free radicals, or for the protection of healthy cells during the cancer treatments that generate oxygen-containing free radicals.

60. Use of a compound selected from diethylfumarate, 5,6-dihydro-2H-pyrane-2-one, beta-ionone, (alpha-bromoisovaleryl)urea, methyl octynoate, monochlorobimane, quercetin and vitamin K-S(II) for the treatment of

diseases caused by ischemia-reperfusion in humans or animals or cellular damage caused by ischemia-reperfusion.

61. Use of a compound selected from diethyl fumarate, 5,6-dihydro-2H-pyran-2-one and beta-ionone for the treatment of stroke in humans or animals.

62. Use of a compound selected from diethylfumarate, alpha-bromoisovalerylurea, methyl octynoate, monochlorobimane, quercetin and vitamin K-S(II) for the treatment or prophylaxis of free-radical damage when ischemia-reperfusion is expected or might occur in humans or animals.

63. Use of a water-based injectable composition containing a compound selected from propyl gallate and 5,6-dihydro-2H-pyran-2-one for the treatment by injection of diseases or cellular damage in humans or animals caused by oxygen-containing free radicals, or of prophylactically preventing or minimising such damage, or of selectively increasing the resistance of normal tissue relative to cancer tissue to free radical damage.

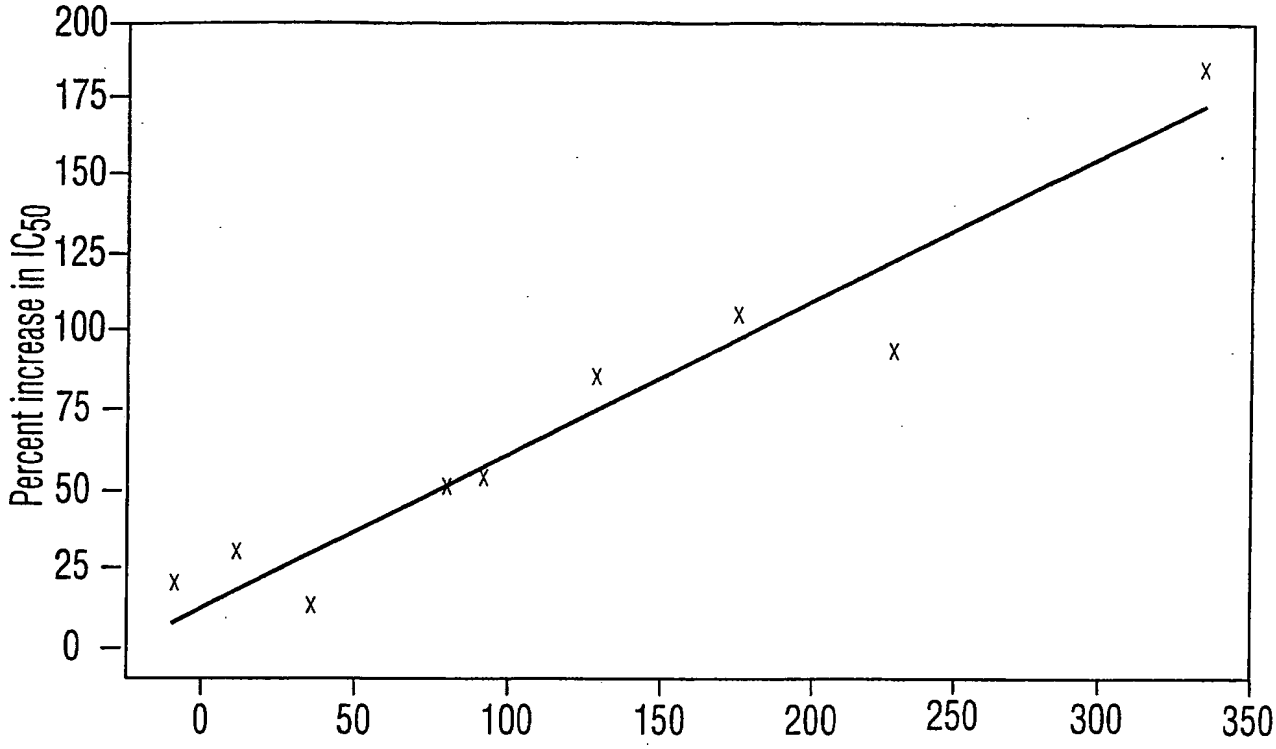

FIG. 1

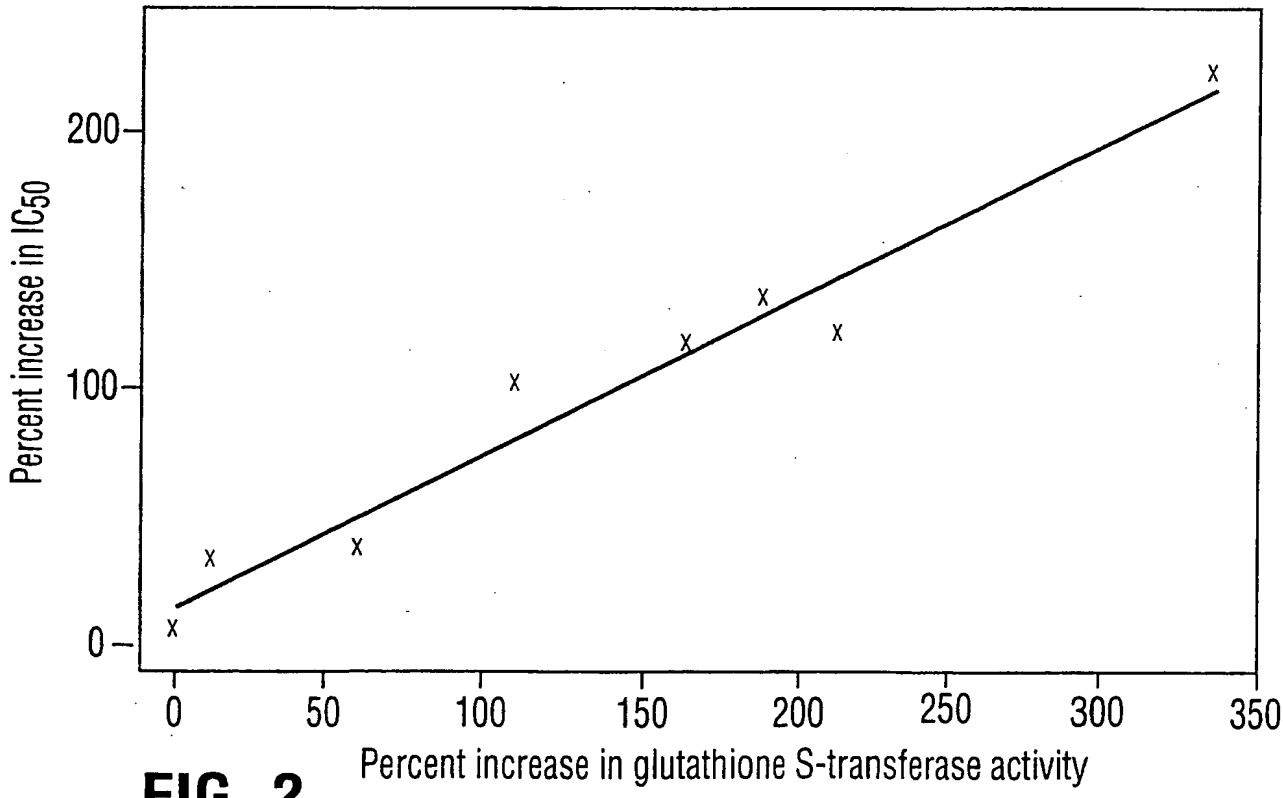

FIG. 2

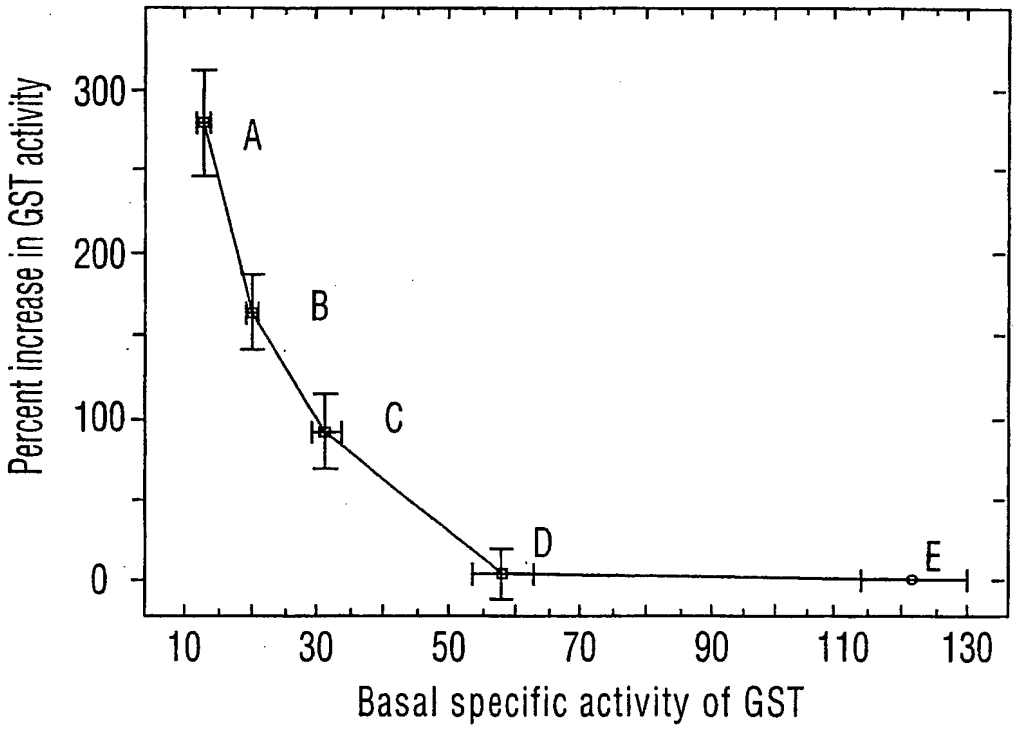

FIG. 3